PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 179, 136–140 (1985)

EVIDENCE FOR A NON-STEROIDAL ANGIOTROPIC FACTOR FROM THE PRIMATE CORPUS LUTEUM: STIMULATION OF ENDOTHELIAL CELL MIGRATION IN VITRO

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Abstract. After luteal cells from 7 midluteal phase cynomolgus monkeys were cultured for 72 h, luteal conditioned media were found to contain angiotropic activity that stimulated endothelial cell migration in vitro, using a 48-microwell chemotaxis assembly. The number of endothelial cells that migrated through 8 μm-pore polycarbonate membranes in 2 h was three-fold greater ($P < 0.01$) with luteal cell-conditioned vs identical unconditioned media. Pre-treatment of luteal cultures with hCG, FSH, or testosterone did not enhance production of the endothelial cell migration stimulating activity ($P > 0.25$). Luteal angiotropic activity was both chemotactic and chemokinetic. Angiotropic activity was retained in steroid-depleted fractions after reversed-phase chromatography. These results demonstrate that monkey luteal cells secrete a non-steroidal factor(s) which directly stimulate(s) migration of endothelial cells in vitro. A luteal angiotropic factor may be an important intraovarian regulator of the formation and lifespan of the primate corpus luteum during the ovarian cycle.

During the ovarian cycle, growth and development of follicles and corpora lutea are accompanied by formation of new intrinsic blood vessels (1). As the corpus luteum forms from the ruptured follicle after ovulation, new capillaries invade the previously avascular membrana granulosa. That is, endothelial cells migrate across the basal lamina, sprout and develop into sinusoidal vessels (2). There is a concomitant involution of blood vessels with follicle atresia or luteolysis. Thus, intraovarian factors that influence angiogenesis may be important regulators of luteinization and luteal lifespan. The present study has used an in vitro bioassay to obtain a direct, objective, and quantitative measure of angiotropic activity from cultured monkey luteal cells.

Materials and Methods. Corpora lutea from mid-luteal phase (day 17-19 of the menstrual cycle) cynomolgus monkeys were dispersed with collagenase (3) and cultured in Falcon 24-well plates (1×10^5 cells/well/ml) for 72 h in Dulbecco's Modified Eagle Medium (GIBCO) supplemented with 25 mm HEPES, 10% fetal bovine serum (FBS; GIBCO), penicillin and streptomycin (GIBCO) and: 1) no hormones; 2) 1.0 IU/ml hCG (Sigma); 3) 100 ng/ml testosterone (Sigma); 4) 100 ng/ml FSH (NIH oFSH-S14); or 5) 1.0 IU hCG + 100 ng/ml testosterone. Endothelial

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0037-9727/85 $1.50

cells were isolated after lightly scraping collagenase-treated thoracic aortas from New Zealand White rabbits and then cultured in CMRL 1066 (GIBCO) supplemented with 25 mM HEPES, 10% calf serum, 10% FBS, 2 mM glutamine, and penicillin/streptomycin. Endothelial cells were subcultured (1:4 split, using 0.05% trypsin-0.02% EDTA) at ∿2 week intervals and then used in the bioassay at the second or third passage. Aortic cells used in the bioassay were tentatively identified as endothelial cells based on three criteria: maintenance of a strict monolayer at confluence; characteristic cobblestone appearance; and positive immunocytochemical staining for angiotensin-converting enzyme (4,5,6).

To test the ability of luteal cell-conditioned media to stimulate migration of endothelial cells in vitro, chemotaxis assays were conducted in 48-microwell modified Boyden chamber assemblies (7, Neuroprobe). Briefly, each bottom well was filled (30 µl) with luteal cell-conditioned or identical unconditioned media and an 8 µm-pore polycarbonate chemotaxis membrane (25 x 80 mm; Nuclepore) was placed on top (8). With the top plate affixed over the membrane, ∿7.5 x 10^4 trypsinized rabbit aortic endothelial cells, suspended in CMRL 1066 containing 0.5% FBS, were aliquoted to each top well. After 2 h incubation at 37°C (5% CO_2), the top of each membrane was wiped over the edge of a rubber blade to remove non-migrated cells. Each membrane was fixed, stained (Diff-Quik), and mounted on a glass slide. To estimate the number of cells that migrated through the membrane into each lower well: each circle of stained cells (8 mm² membrane area/well) was centered below the 10X objective; cells wholly within an eyepiece reticle grid (0.64 mm² membrane area) were counted and the sum converted to cells/mm². Statistical comparisons were performed by analysis of variance and multiple range tests.

Results. Media conditioned by monkey luteal cells markedly stimulated migration of endothelial cells (Fig. 1). The number of endothelial

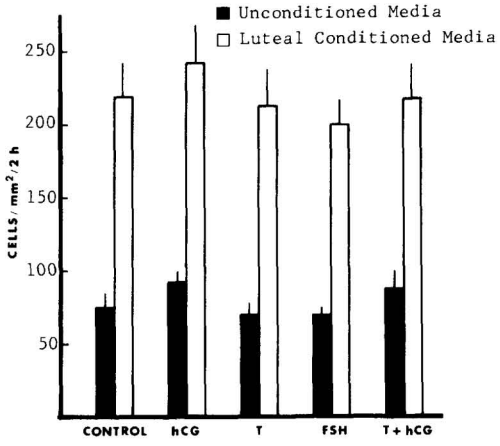

Fig. 1. Migration rate of endothelial cells from top chambers, containing ∿7.5 x 10^4 cells/well in CMRL 1066 + 0.5% FBS, through 8 µm-pore polycarbonate membranes to bottom chambers containing luteal conditioned media (open bars) or identical unconditioned media (closed bars). Individual samples were assayed in adjacent triplicate wells. Height of each bar is the average (+ SE) of 7 monkeys. CONTROL refers to media not supplemented with testosterone (T), FSH, or hCG during luteal cell cultures.

cells that migrated through 8 µm-pore polycarbonate membranes within 2 h was three-fold greater when luteal cell-conditioned media (open bars) vs identical unconditioned media (closed bars) were in bottom wells ($P < 0.01$). Hormone treatments during luteal cell culture had no additional augmenting effect on luteal production of angiotropic activity ($P > 0.25$). Conditioned and unconditioned media were fractionated using reversed-phase C_{18} columns (Baker) to remove steroids. Activity in the steroid-depleted fraction was decreased but was not different ($P > 0.2$) from unfractionated medium (Fig. 2). Steroid fractions reconstituted with unconditioned media were not more active than unconditioned media alone.

To determine whether the migration stimulating activity from luteal cells represented chemotaxis or chemokinesis, each combination of unconditioned or luteal cell-conditioned medium in top and bottom wells of the chamber was used in the

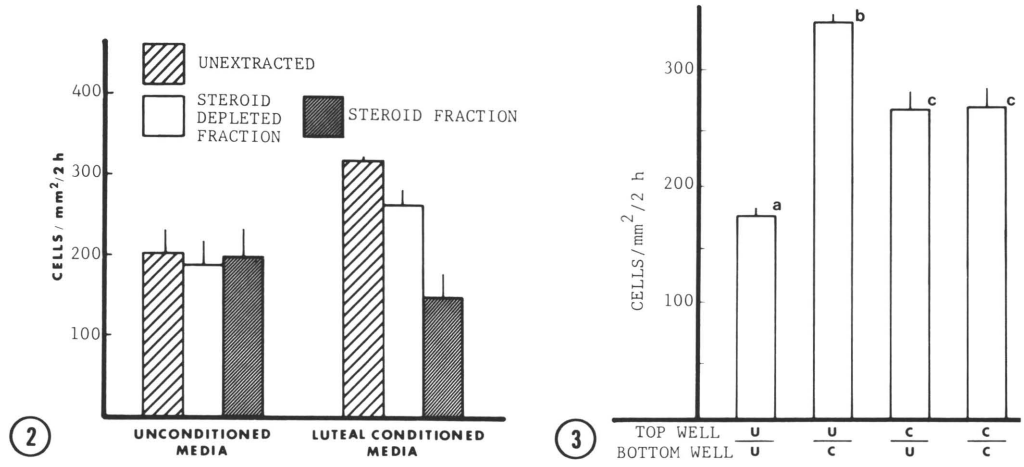

Fig. 2. Endothelial cell migration in response to media fractionated by elution through C_{18} reversed-phase columns which retain >95% of the steroids. After eluting with methanol and drying under vacuum, steroid fractions were reconstituted in unconditioned medium. Each bar represents the mean (+ SE) based on triplicate determinations for 4 monkeys.

Fig. 3. Demonstration of both chemotactic and chemokinetic effects of luteal conditioned (C) vs unconditioned (U) media on endothelial cell migration. Each bar depicts the composite mean (+ SE) of triplicate wells for 6 monkeys; bars with different superscripts are different ($P < 0.01$).

bioassay (Fig. 3). That is, a 2 x 2 factorial design was used to distinguish between endothelial cell movement in response to a concentration gradient (chemotaxis) and stimulation of cell motility per se, (chemokinesis). As depicted in Fig. 3, a chemokinetic component, which increased cell migration from about 170 cells/mm²/2 h (U/U) to about 260 cells/mm²/2 h (C/C), was observed in the absence of a concentration difference across the membrane. Endothelial cell motility was increased to a comparable degree when conditioned media were present only in upper wells (C/U). The greatest stimulation, to about 320 cells/mm²/2 h, occurred in the U/C arrangement, which indicates a chemotactic stimulation beyond chemokinesis (i.e., U/C > C/C or C/U).

Discussion. These results demonstrate that monkey luteal cells release a nonsteroidal factor(s) that markedly stimulates endothelial cell migration in vitro. This activity meets criteria of an angiogenic factor (9,10): stimulation of endothelial cell migration is direct, i.e. not mediated by other cell-types; endothelial cell response is rapid (up to three-fold increases in 2 h); stimulation is chemotactic not just chemokinetic, i.e. not only is endothelial cell motility increased, net cell movement is greatest up a concentration gradient. Although nonspecific conditioning of media cannot be excluded, the nature of responses observed provides circumstantial evidence for luteal cell secretion of a specific angiotropic substance(s).

Our findings with monkey luteal cells extend earlier observations by others (11,12) of luteal angiogenesis in nonprimate species; more recent studies, using in vivo and in vitro bioassays (13,14), indicate follicular angiogenic activity in the ovary, as well. Although in vivo studies are needed to confirm angiogenic activity of the monkey corpus luteum, the in vitro bioassay employed here has the advantages of demonstrating direct endotheliotropic action, and of

providing objective and quantitative criteria of angiotropic effects. The microwell Boyden chamber technique not only permits distinction between chemotaxis and chemokinesis (8,15), it also allows rapid screening of samples for angiotropic activity.

In summary, demonstration of angiotropic activity in media conditioned by monkey luteal cells suggests that the primate corpus luteum secretes in vivo an angiogenic factor that may be important in regulating its formation and lifespan. These kinds of findings underscore the importance of intraovarian mechanisms (16) in the control of the ovary's cyclic structures and, in turn, the primate ovarian cycle (17).

Acknowledgments. The authors are indebted to Dr. P.R.B. Caldwell for generously supplying goat anti-rabbit angiotensin-converting enzyme antiserum, to Dr. B. M. Glazer for helpful suggestions on the chemotaxis assay, and to J. Brown, C. Quigley, and C. Shaw for preparing the typescript. Supported by NIH grants HD14573 and HD17171.

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Received February 19, 1985.
P.S.E.B.M. 1985, Vol. 179.
Accepted March 19, 1985.